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Qi Cao

E-Mail:

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**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

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**14. ABSTRACT**

Our preliminary data strongly suggest that BMI1 is a master regulator of castration-resistant prostate cancer (CRPC) progression. Our objective is to determine how BMI interacts with epigenetic complexes and with AR to regulate tumor suppressor gene expression. We aim to identify novel binding partners and regulators of oncogene expression, which will lead to a better understanding of AR signaling and dysfunction. Specifically, we will identify how BMI1 and PRC1 proteins mediate their oncogenic functions by recruiting AR and distinct binding partners to promote castration-resistance of PCa. Furthermore, we will evaluate the therapeutic efficacy of targeting BMI1 and of combinational targeting of BMI1 and AR in castration-resistant prostate cancer. During the first year of this project, we discovered that BMI1 directly binds to Androgen Receptor and prevents it from MDM2-mediated protein degradation. We further demonstrated that inhibiting BMI1 decreased prostate cancer tumor growth in VCaP murine xenograft.

**15. SUBJECT TERMS:** **Polycomb, BMI1, Androgen Receptor, ubiquitination, PRC1, castration-resistant prostate cancer**

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## Table of Contents

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## INTRODUCTION

Each year, over 240,000 American men are diagnosed with prostate cancer (PCa). B lymphoma Mo-MLV insertion region 1 homolog (BMI1) have been shown associating with metastatic prostate cancer by cDNA microarray analyses and tissue microarray analysis. BMI1 is an epigenetic component of a Polycomb Repressive Complex 1 (PRC1), maintaining gene repression. We have demonstrated that BMI1 promotes prostate cancer progression by repressing multiple tumor suppressors. However, its precise role in castration-resistant prostate cancer (CRPC) remains unclear. Our preliminary data strongly suggest that BMI1 is a master regulator of castration-resistant prostate cancer (CRPC) progression. Our objective is to determine how BMI interacts with epigenetic complexes and with AR to regulate tumor suppressor gene expression. We aim to identify novel binding partners and regulators of oncogene expression, which will lead to a better understanding of AR signaling and dysfunction. Specifically, we will identify how BMI1 and PRC1 proteins mediate their oncogenic functions by recruiting AR and distinct binding partners to promote castration-resistance of PCa. Furthermore, we will evaluate the therapeutic efficacy of targeting BMI1 and of combinational targeting of BMI1 and AR in castration-resistant prostate cancer.


## KEYWORDS

BMI1, Prostate Cancer, Polycomb Repressive Complex, Androgen Receptor, Castration-Resistant Prostate Cancer, small molecule inhibitor

## ACCOMPLISHMENTS

### A. What were the major goals of the project?

| | Months | Percentage of completion |
|---|---|---|
| **Major Task 1: to elucidate the mechanism by which BMI1 interacts with AR and recruits AR** | 1-24 | 80% |
| **Major Task 2: to dissect how BMI1 plays its role in androgen signaling** | 1-36 | 50% |
| **Major Task 3: to evaluate BMI1 as a therapeutic target for advanced prostate cancer patient treatment.** | 1-36 | 50% |
| **Milestone(s) Achieved: discovery of critical domains for AR and PRC1 protein interactions; determination of binding affinity of AR and PRC1, and set-up of a high-throughput platform for small molecule inhibition screening** | 24 | 60% |
| **Milestone(s) Achieved: identification and characterization of novel binding partners and downstream targets of BMI1 and AR in androgen-dependent and -independent PCa cells, in the presence and absence of androgen.** | 36 | 40% |
| **Milestone(s) Achieved: evaluation of BMI1 as a therapeutic target for CRPC patients and rationale for combinatorial targeting of AR and BMI1 in clinic trials; publication of 1-2 peer reviewed papers** | 36 | 40% |

### B. What was accomplished under these goals?

1. **BMI1 binds to AR N-terminal domain**.

Androgen receptor (AR) has 3 functional domains, N-terminal domain (NTD), DNA binding domain (DBD) and ligand binding domain (LDB), and we generated 3 AR truncated mutants containing these 3 domains respectively (**Fig. 1A**). When we overexpressed these 3 Halo-tagged AR mutants or full-length AR in HEK-293T cells, followed by pull-down with anti-BMI1 or Halo-binding ligand using these cell lysates. As shown in **Fig. 1B**, IP with anti-BMI1 pulled down full-length and AR-NTD, but not AR-DBD or AR-LBD. On the other hand, pulldown with Halo ligand (AR or AR mutants) showed that only full-length AR and AR-NTD bound to BMI1 while AR-DBD or AR-LBD did not (**Fig. 1C**). All these results suggest that AR NTD domain is essential for BMI1 and AR interaction.

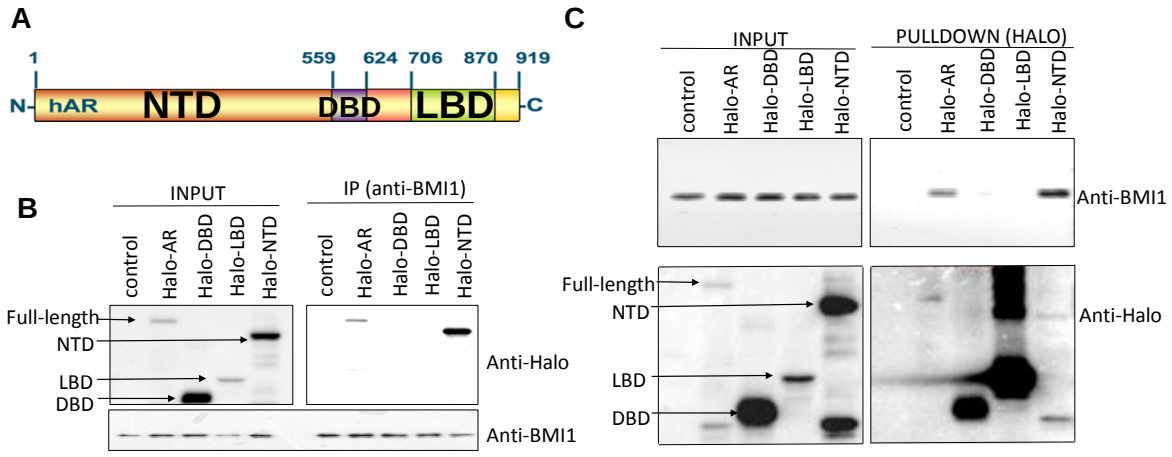


**Figure 1. BMI1 binds to AR N-Terminal Domain.**

(**A**) Functional domains of AR. (**B, C**) Pull-down assay of HEK-293T cell lysates ectopically overexpressing Halo-tagged full-length AR, truncated mutants AR-NTD, AR-DBD or AR-LBD, along with BMI1, with anti-BMI1 (**B**) or Halo-ligands (**C**) magnetic beads followed by IB analysis with anti-Halo (to detect AR or mutants) and anti-BMI1 confirmed the AR and BMI1 interaction.

**2. BMI1 prevents AR from MDM2-mediated protein degradation.**

Next, to explore the function of BMI1 in prostate cancer, we knocked down BMI1 by BMI1 specific siRNA duplexes or shRNAs in multiple prostate cancer cell lines. Surprisingly, AR and its downstream targets, such as PSA were decreased at protein levels by BMI1 knockdown (**Fig. 2A and B**). However, AR transcript levels were not altered by BMI1 depletion while PSA transcript levels were decreased by BMI1 knockdown (data not shown). The newly identified BMI1 inhibitor PTC-209 also decreased AR and PSA levels (**Fig. 2C**). We anticipated that BMI1 may prevent AR protein from degradation. To test this hypothesis, we treated C4-2 cells with PTC-209 and then treated cells with the proteasome inhibitor MG132, lysosome inhibitors NHCl or Chloroquine. As shown in **Fig. 2D**, while co-treatment of $NH_4Cl$ or Chloroquine did not show any significant effects, co-treatment of MG132 rescued PTC209-mediated downregulation of AR, but did not affect the PTC209-induced BMI1 decrease, suggesting that depletion of BMI1 destabilizes and degrades AR through proteasomes. Furthermore, to examine if BMI1 depletion-mediated degradation is the major cause of AR downregulation, we treated our BMI1 stable knockdown C4-2 and control C4-2 cells with cycloheximide, a protein synthesis inhibitor, and measured the AR protein levels at various time points by immunoblot analysis. As shown in **Fig. 2E**, the half-life of AR protein was remarkably reduced from 16-17 hours to 5 hours in BMI1 knockdown cells compared to control cells, suggesting that the decrease in AR protein levels by inhibiting BMI1 is due to post-translational degradation. Similarly, AR protein half-life was also significantly reduced to 4 hours in the PTC-209-treated C4-2 cells compared to vehicle (DMSO)-treated C4-2 cells (**Fig. 2F**).

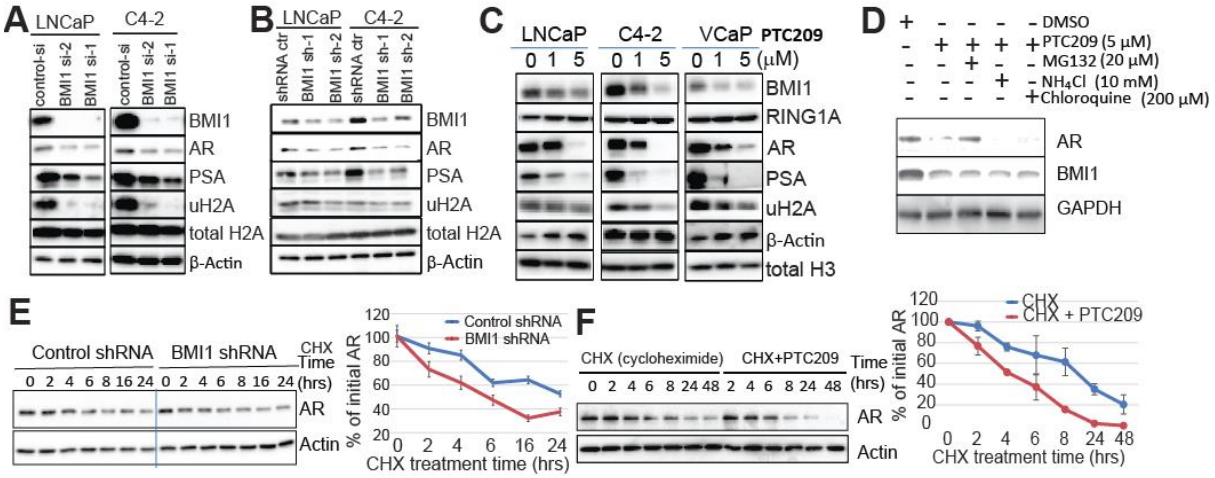


**Figure 2. BMI1 regulates AR protein stability.**

**(A, B)** Immunoblot analysis shows that depletion of BMI1 by siRNA duplexes **(A)** or by shRNA stably knocking down **(B)** decreased BMI1, uH2A levels, along with AR and PSA levels. (**C**) 24-hour treatment of BMI1 inhibitor PTC209 decreased BMI1 protein levels along with AR, PSA and uH2A levels at the indicated concentrations. (**D**) C4-2 cells were treated with PTC209 along with indicated drugs for 8 hours, followed by immunoblot analysis with the indicated antibodies. MG132, proteasome inhibitor; $NH_4Cl$ and Chloroquine, lysosome inhibitors. MG132 blocked PTC-209 induced AR degradation. **(E, F)** BMI1 stable knockdown C4-2 and control cells (**E**) or PTC209 (or DMSO control) treated C4-2 cells (**F**) were treated with 50 μg/ml cycloheximide (protein biosynthesis inhibitor). Cells were harvested at the indicated time points and immunoblot analysis with anti-AR and anti-β-actin antibodies on the same membrane (left panel). Density of each band was analyzed by ImageJ and normalized respectively to β-actin (loading control). Each value is the average of 3 independent experiments. Error bars represent ±S.E.M. In this figure, total H2A, H3, GAPDH and β-actin served as loading controls. These data represent at least three biological replicates of IB analyses.

To confirm that BMI1 plays a role in AR stability and degradation, we treated C4-2 cells with PTC-209 to induce AR ubiquitination, but co-treatment of MG132 prevented AR degradation, and then we overexpressed BMI1 or control in the treated C4-2 cells. As shown in **Fig. 3A**, BMI1 overexpression remarkably decreased the PTC-209-induced ubiquitinated AR levels compared to control treatment. It has been reported that several ubiquitin E3 ligases, such as MDM2, CHIP and SPOP, could ubiquitinate and degrade AR [46, 47]. Since both MDM2 and BMI1 bind to the AR NTD domain, we hypothesized that BMI1 may inhibit MDM2-AR interaction by competition binding, and then protect AR from degradation. To test our hypothesis, we co-transfected AR+BMI1 or AR+MDM2 into HEK293T cells and immunoprecipitated AR. As shown in **Fig. 3B**, overexpression of BMI1 decreased AR-immunoprecipitated MDM2, while overexpression of MDM2 decreased AR-immunoprecipitated BMI1, confirming our hypothesis that BMI1 and MDM2 competitively bind to AR. Importantly,

the BMI1 depletion-mediated AR downregulation was rescued by two MDM2 siRNA duplexes (**Fig. 3C**), demonstrating that BMI1 protects AR from MDM2-mediated degradation.

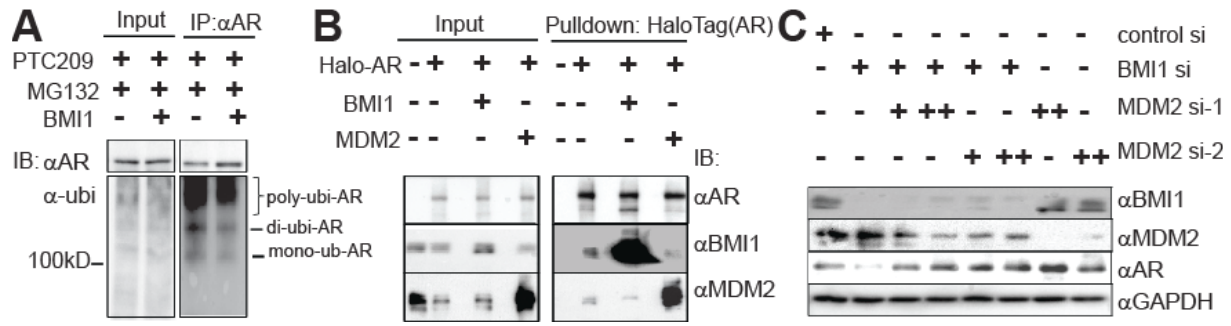


**Figure 3. MDM2 is essential for BMI1 loss-mediated AR degradation.**
**(A)** C4-2 cells were treated with PTC209 and infected by BMI1 adenovirus (or control virus) for 24 hours. Post 8-hour 20μM MG132 treatment, immunoprecipitation was performed using the cell lysates with ant-AR antibody, followed by immunoblot analysis with anti-AR and anti-ubiquitin antibodies. Overexpression BMI1 decreased ubiquitinated AR. **(B)** HEK-293T cells were transfected with the indicated plasmids. Cells were lysed after 48 hours, and pull-down assay was performed with Halo-Tag magnetic agarose beads, followed by immunoblot analysis with anti-AR, BMI1 and MDM2 antibodies. Overexpression of BMI1 (or MDM2) decreased AR-MDM2 (or AR-BMI1) interaction. **(C)** C4-2 cells were transfected with indicated siRNAs. 72 hours post-transfection, immunoblot analysis was performed with indicated antibodies. Knockdown MDM2 blocked BMI1-loss induced AR decrease.

**3. BMI1 occupies AR target upstream regions and regulates their expression levels.** Next, when we performed ChIP-qPCR analysis with anti-BMI1, uH2A, and AR antibodies using BMI1 stable knockdown C4-2 (which grew more slowly than control cells) and control cells, we observed that BMI1 and uH2A, along with AR, were enriched at the upstream regions of known AR targets Furthermore, the enrichments were decreased in BMI1 knockdown cells compared to control cells (**Fig. 4A**). Real-time qPCR analysis also showed that known AR-activated genes KLK3 (PSA) and TMPRSS2 were downregulated, while AR-repressed genes MET and SI were upregulated (**Fig. 4B**). ChIP-qPCR and gene expression qPCR analysis using PTC209 and vehicle (DMSO)-treated C4-2 and LNCaP cells were consistent with these findings. ChIP-Seq analysis also demonstrated that 20% and 46% of BMI1-enriched regions (in sh-Control and sh-BMI1 cells, respectively) were also enriched with AR (**Fig. 4C**), suggesting that BMI1 and AR have both shared and unique target genes. Importantly, knocking down BMI1 remarkably decreased the enrichment of AR in its target loci (**Fig. 4D**). The total number of AR enriched regions was also decreased by BMI1 knockdown (**Fig. 4E**). A sample genome browser view of AR target, The AR enrichment at KLK3 region was shown as a sample in **Fig. 4F**.

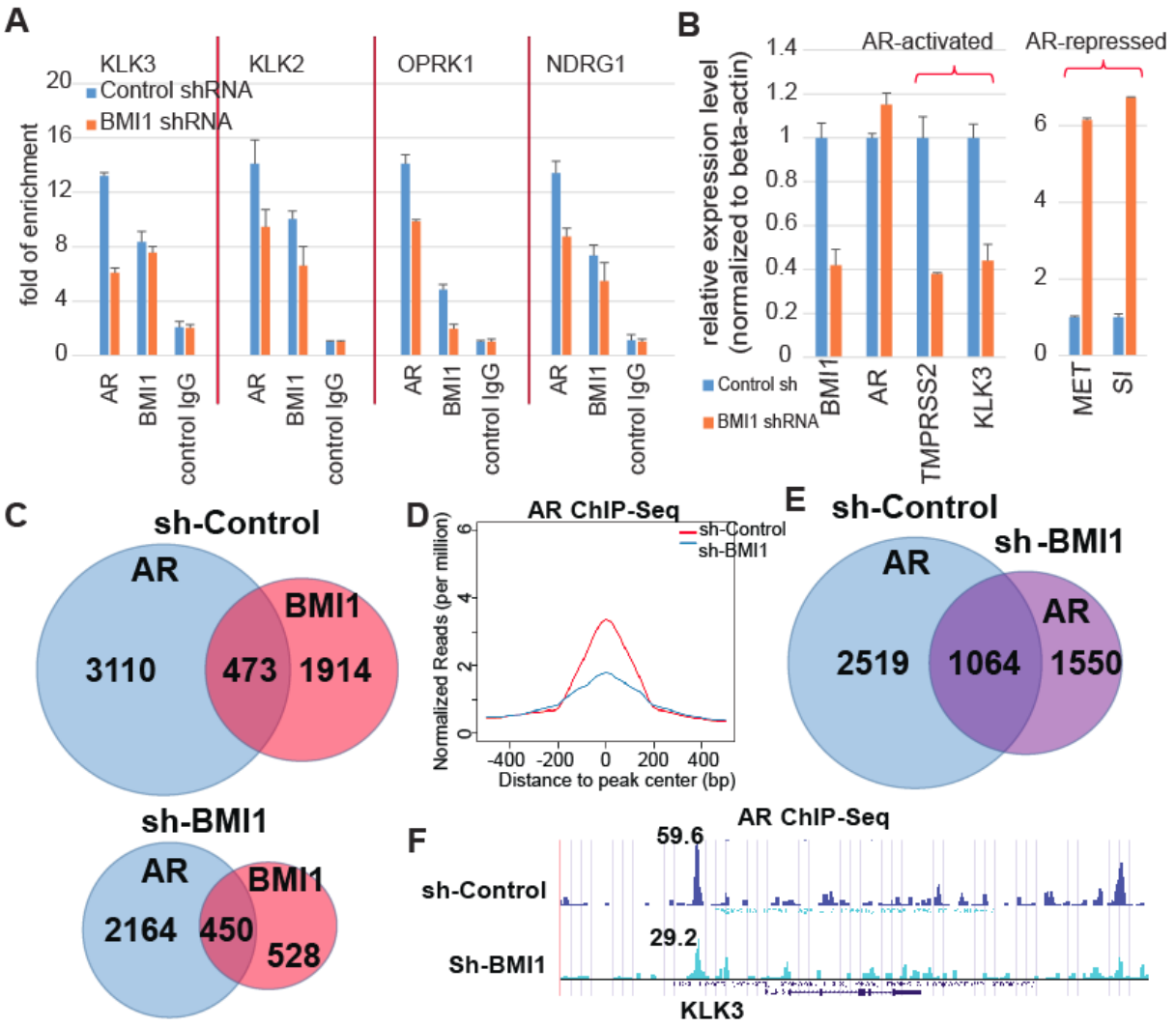


**Figure 4. BMI1 is enriched to AR target upstream regions and regulates AR targets.** **(A)** ChIP was performed with anti-AR and BMI1 (normal IgG as a negative control) using BMI1 stable knockdown and control shRNA C4-2 cells, following qPCR analysis with known AR target upstream regions. **(B)** qPCR gene expression analysis of *BMI1*, *AR*, androgen-stimulated genes *KLK3* and *TMPRSS2*, and androgen-repressed genes *MET* and *SI* in BMI1- stable knockdown and control shRNA C4-2 cells. **(C)** Venn diagrams showing the shared and unique BMI1 and AR target genes in control or BMI1 stable knockdown C4-2 cells. 20-46% of BMI1 regulated genes are AR targets (in sh-Control and sh-BMI1 C4-2 cells, respectively). **(D)** Genome-wide AR enrichment in C4-2 cells stably expressing scramble shRNA (red) or sh-BMI1 (blue) at all annotated gene promoters. **(E)** Venn diagrams shows that knockdown of BMI1 reduces total amount of AR enriched regions, and also shifts AR to new binding loci. **(F)** Example IGV browser views for AR ChIP-seq in C4-2 cells stably expressing either scramble shRNA or sh-BMI1. The peak height decreased (~ two fold, numbers in blue) in BMI1 knock down samples in genomic regions such as KLK3 (PSA).

**4. BMI1 inhibitor PTC209 and AR antagonist MDV3100 synergistically inhibited the growth of AR+ PCa.** Cell proliferation assay (**Fig. 5A, B**, 72 hours drug treatment) showed that PTC209 alone, at low IC50 concentrations (< 1μM), decreased the proliferation of C4-2 and LNCaP cells. These data suggest that BMI1 inhibitors may be applicable for treatment of PCa. In addition, as shown in Fig.10A and B, the presence of 2.5 μM MDV3100 remarkably decreased the IC50 of PTC209 in LNCaP and C4-2 cells. More importantly, median dose effect and isobologram analyses showed that co-treatment with PTC209 and MDV3100 synergistically inhibited the proliferation of AR+, but not PC3, AR-negative PCa cells (**Fig. 5C-F**). Combination index (CI) was calculated using Calcusyn software for each cell line. All CI values were less than 1.0, suggesting a synergistic effect between PTC209 and MDV3100.

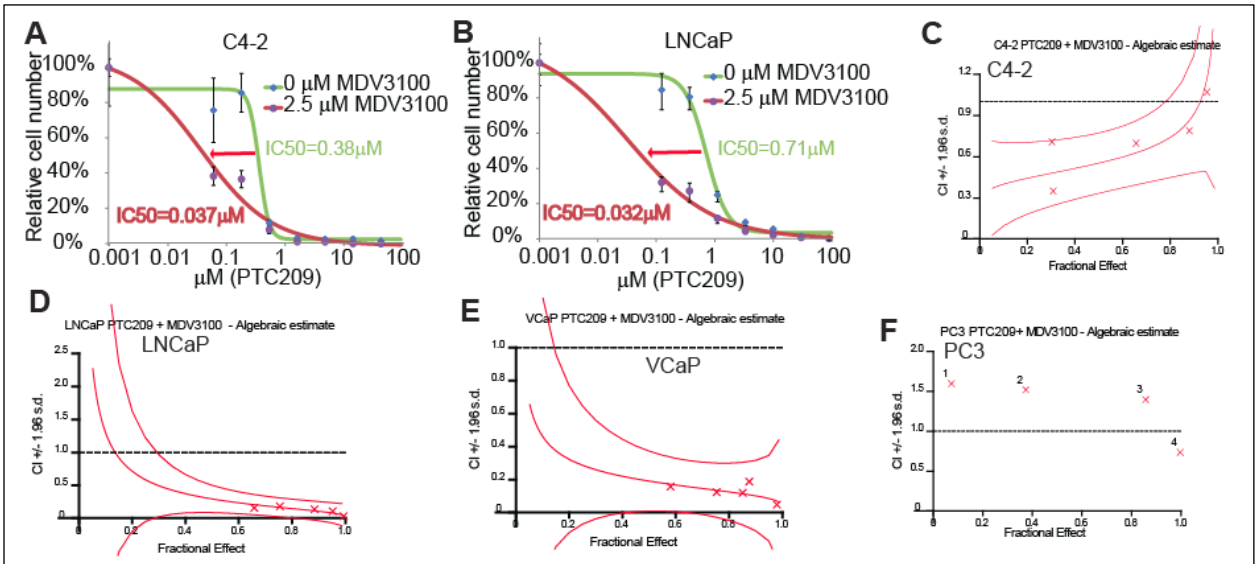


**Figure 5. BMI1 inhibitor PTC-209 synergistically inhibits AR+ PCa cell growth with MDV3100 at a very low IC50 concentration. (A, B)** C4-2 and LNCaP cells were cultured in 96-well plates and treated with BMI1 inhibitor PTC-209 at the indicated concentrations with or without 2.5μM MDV3100 for 72 hours, followed by CellTiter-Glo cell proliferation assay. IC50 of PTC209 was decreased 10-fold by 2.5μM MDV3100 compared to control treatment. (**C-F**) C4-2, LNCaP, VCaP and PC3 cells were treated with PTC209 and MDV3100 for 72 hours, followed by CellTiter-Glo cell proliferation assay. Median dose and isobologram analyses were performed using Calcusyn. CI (combination index) values <1.0 suggest synergism between PTC209 and MDV3100.

**5. BMI1 inhibitor PTC209 decreased VCaP xenograft tumor growth.** More importantly, our two independent VCaP murine xenograft assays demonstrated that PTC209 treatment significantly reduced established VCaP tumor growth, as well as Enzalutamide treatment (**Fig. 6A**). The toxicitiy of combination treatment of PTC209 and Enzalutimide were also evaluated in SCID mice. Mice treated with this combination did not show loss of body weight (**Fig. 6B**), and there was no sign of toxicity after 3 weeks treatment.

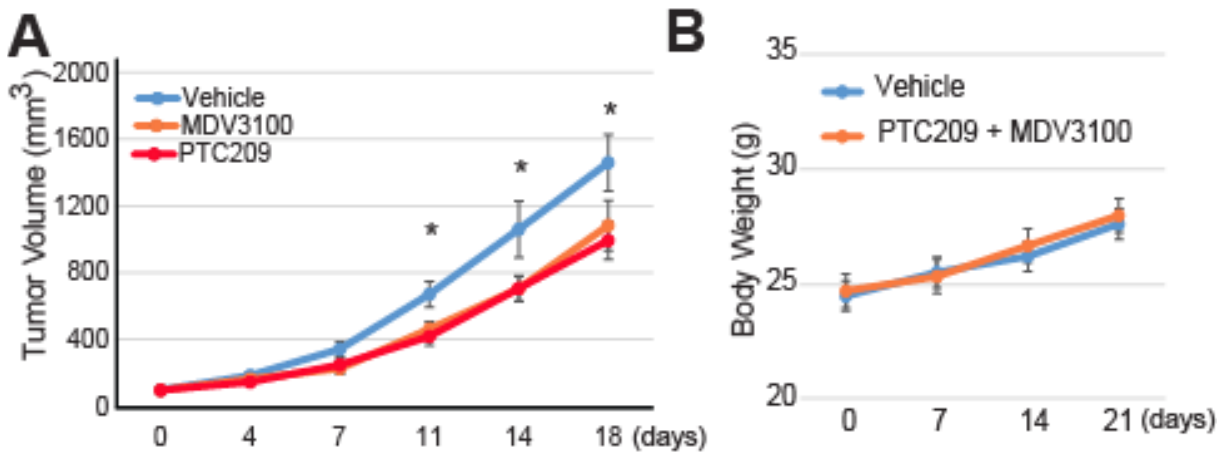


**Figure 6. BMI1 inhibitor PTC-209 remarkably decreased PCa tumor growth.**

**(A)** VCaP cells were implanted into left flanks of 6-week old male SCID mice. Tumors upon reaching volume of 100mm$^3$ were subjected to treatment with vehicle control (CTRL, n=13), PTC209 (60mg/kg, n=11) alone or MDV3100 (10 mg/kg, n=12) I.P. 5 times per week. Mice were closely monitored and weighed every day. *$p<0.05$ (student t-test, between vehicle and PTC209). **(B)** Male SCID mice were treated with combo (PTC209, 60mg/kg + MDV3100, 10mg/kg) 5 times per week for 3 weeks or vehicle control. Mice were closely monitored and weighed every week.

**C. What opportunities for training and professional development has the project provided?**

**Honor**

2016-2019, American Cancer Society Research Scholar Award

**New adjunct faculty positions**

9/2016-present, adjunct assistant professor, Weill Cornell Medical College, New York, NY

9/2016-present, adjunct member, Comprehensive Cancer Center, Baylor College of Medicine, Houston, TX

**National conferences attended**

Oct. 7-10, 2015, 22nd Annual Prostate Cancer Foundation Scientific Retreat, Washington D.C.

April 16 - 20, 2016, AACR Annual Meeting, New Orleans, LA

DoD PCRP IMPaCT 2016 Young Investigators Meeting. August 4-5, 2016, Baltimore, MD

**D. How were the results disseminated to communities of interest?**

Nothing to Report.

**E. What do you plan to do during the next reporting period to accomplish the goals?**

We are continuously working on this project and pursue the aims. We are completing the proposed ChIP-Seq and RNA-Seq analyses using BMI1 knockdown cells to examine if AR recruitments are altered and if AR downstream targets are dysregulated by BMI1 depletion. In addition, we are working on tissue microarray analysis to investigate if BMI1 and AR protein levels are correlated during prostate cancer progression.

Besides the VCaP xenograft assays reported here (Fig. 6), we are evaluated if BMI1 inhibitor PTC-209 could inhibit VCaP and 22RV1 tumor growth in the pre-castrated mice. In addition, we are evaluating if the combination treatment of PTC-209 and enzalutamide is better than single agent.

## IMPACT

**A. What was the impact on the development of the principal discipline(s) of the project?**
*Nothing to Report*

**B. What was the impact on other disciplines?**
*Nothing to Report*

**C. What was the impact on technology transfer?**
*Nothing to Report*

**D. What was the impact on society beyond science and technology?**
*Nothing to Report*

## CHANGES/PROBLEMS

**A. Changes in approach and reasons for change**
*Nothing to Report*

**B. Actual or anticipated problems or delays and actions or plans to resolve them**
*Nothing to Report*

**C. Changes that had a significant impact on expenditures**
*Nothing to Report*

**D. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
*Nothing to Report*

**E. Significant changes in use or care of human subjects**
*Nothing to Report*

**F. Significant changes in use or care of vertebrate animals.**
*Nothing to Report*

**G. Significant changes in use of biohazards and/or select agents**
*Nothing to Report*

**PRODUCTS:**

**A. Publications, conference papers, and presentations**

**a. Journal publications.**

*Nothing to Report*

**b. Books or other non-periodical, one-time publications.**

*Nothing to Report*

**c. Other publications, conference papers, and presentations.**

Poster presentation

1) Jungsun Kim, Weihua Jiang, Irfan A. Asangani, Arul M. Chinnaiyan, Qi Cao. The role of EED in histone modification and prostate cancer. The 22nd Annual Prostate Cancer Foundation Scientific Retreat. Oct. 7-10, 2015, Washington D.C.

2) Sen Zhu, Jungsun Kim, Bingnan Gu, Weihua Jiang, Lin Yan, Ladan Fazli, Jonathan Zhao, Xuesen Dong, Jindan Yu, Qi Cao. A Novel Role of BMI1 in Androgen Receptor Pathway. DoD PCRP IMPaCT 2016 Young Investigators Meeting. August 4-5, 2016, Baltimore, MD

B. **Website(s) or other Internet site(s)**

*Nothing to Report*

C. **Technologies or techniques**

*Nothing to Report*

D. **Inventions, patent applications, and/or licenses**

*Nothing to Report*

E. **Other Products**

*Nothing to Report*

## PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

### A. What individuals have worked on the project?

| | |
|---|---|
| Name: | *Qi Cao* |
| Project Role: | *PI* |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 2 |
| Contribution to Project: | *Conceive the idea, lead the project, design experiments and analyze the data* |
| Funding Support: | *DoD PCRP IDA, Prostate Cancer Foundation, American Cancer Society, Start-up* |

| | |
|---|---|
| Name: | *Sen Zhu* |
| Project Role: | *Post-Doctoral* |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 4 |
| Contribution to Project: | *Perform major experiments and analyze the data* |
| Funding Support: | *DoD PCRP IDA, Prostate Cancer Foundation, American Cancer Society, Start-up* |

| | |
|---|---|
| Name: | *Weihua Jiang* |
| Project Role: | *Post-Doctoral* |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 6 |
| Contribution to Project: | *Perform xenograft experiments and analyze the data* |
| Funding Support: | *Prostate Cancer Foundation, American Cancer Society, Start-up* |

| | |
|---|---|
| Name: | *Lin Yan* |

| | |
|---|---|
| Project Role: | *Graduate Student* |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 5 |
| Contribution to Project: | *Help Drs. Zhu and Jiang perform xenograft experiments and molecular and cellular assays, and analyze the data* |
| Funding Support: | *Prostate Cancer Foundation, American Cancer Society, Start-up* |

B. **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

C. **What other organizations were involved as partners?**

1. Collaborator Names: Jindan Yu and Jonathan Zhao
   **Organization Name:** Northwestern University Feinberg School of Medicine
   **Location of Organization:** Chicago, IL, USA
   **Partner's contribution to the projec**t: **Collaboration**

2. Collaborator Names: Xuesen Dong and Ladan Fazli
   **Organization Name:** Vancouver Prostate Centre and Department of Urologic Sciences, University of British Columbia
   **Location of Organization:** Vancouver, BC, Canada V6H 3Z6
   **Partner's contribution to the projec**t: **Collaboration, Facilities**